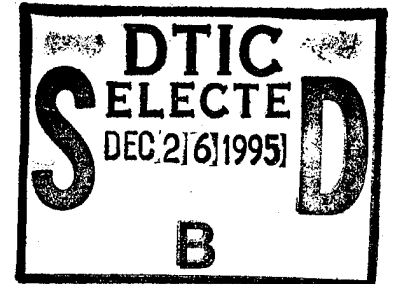AD_____

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PRINCIPAL INVESTIGATOR:

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CONTRACTING ORGANIZATION:

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13. ABSTRACT *(Maximum 200 words)*

In this U.S. Army sponsored research program, we proposed to identify and characterize the serum factor(s) which regulated estrogen-responsive breast cancer cell growth and to conduct another study to ask if loss of serum factor(s) control was related to hormone autonomy. During the first year, we have been successful beyond our expectations. We have demonstrated that two well known plasma glycoproteins, sex hormone-binding globulin (SHBG) and corticosteroid binding globulin (CBG) are negative regulators of steroid hormone responsive growth and that estrogens and progesterone reverse this inhibition. Furthermore, steroid hormone autonomous cells appear not to be regulated by either SHBG or CBG. Our protein chemistry data show that the SHBG we have isolated is not the common (Type I) form most often studied, but a variant that has only 30 to 40% amino acid sequence homology. The results to date suggest that breast cancer growth may be regulated by a new form of SHBG which we have designated Type II. The search for human Type II SHBG will be a major topic of this research for the next year.

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I. TABLE OF CONTENTS

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II. INTRODUCTION

A. Nature of the Problem

During the first 12 months of our US Army Breast Cancer Program project, we have successfully addressed one of the major unresolved issues in breast cancer endocrine physiology. In our original proposal, we planned first (TASK 1) to identify the serum factor(s) which regulated estrogen-dependent breast cancer cell growth. We are pleased to report that two exceptionally significant serum factors have been identified. They both are well known human plasma glycoproteins. Both are related to the action of sex steroids or progestins. During the first year of this project, we purified these serum factors from horse and rat serum and characterized them by several different methods including partial amino acid sequencing.

B. Background

This project is based on recent discoveries in our laboratory which have resolved a longstanding problem in breast cancer endocrine physiology. In 1896, Beatson reported that oophorectomy had palliative effects on breast cancer (1). That report first implicated estrogens and progestins in breast cancer growth. Since that time, researchers have identified specific nuclear receptors for these steroid hormones (2-3) and characterized them by cDNA cloning and many other methods (4). This advance led to the assumption that the interaction of estrogens and progestins with their specific nuclear receptors was sufficient to explain target tissue growth (2-3). However, as work progressed, this point of view became less defensible.

Many investigators came to the conclusion that other regulatory molecules also were required. For example, our laboratory proposed that plasma contained an estrogen regulated mediating "estromedin" which controlled target cell proliferation (5). Several putative estromedins were purified from tissues and identified as insulin-like growth factors I and II (IGF-I and IGF-II) (6,7), transforming growth factor α (TGFα) (8), and fibroblast growth factors (9). None proved to be related to estrogen dependent breast cancer cell growth (10). Another laboratory proposed that plasma contained an inhibitor (estrocolyone) which negatively controlled target tissue growth (11). It has been proposed that estrocolyone inhibits growth by binding to target cells, and that this restraint is reversed when steroid hormones associate with the inhibitor to release it from cells. Estrocolyones have proven to be unstable during isolation and because of this have not been characterized at the molecular level (12). Another group concluded that serum albumin was "estrocolyone" (13). This hypothesis was not confirmed (10). Continuing with the theme of serum factor regulation, other investigators did not offer specific names for the agents involved, but did conclude that serum contained a critically important regulator(s) which had not yet been identified (14-17). In support of this viewpoint, it was known that estrogen mitogenic effects *in vitro* were difficult to demonstrate (17-20), especially in completely serum-free hormonally defined medium (10,21,22). When estrogen mitogenic effects were found *in vitro*, they were most pronounced in serum supplemented cultures (11,17,23-26). Recently, we found that the ferric iron in culture medium was deleterious to hormone-responsive growth (27). Use of "low-iron" medium now

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permits identification of very large estrogen mitogenic effects in serum-supplemented culture (27).

The work of Sporn and Todaro (28) opened new possibilities with regard to growth factors and cancer. They proposed that growth factors might act as autocrine, paracrine or endocrine regulators. Many groups joined in these studies. For example, one consortium of investigators proposed that estrogens induced TGFα , IGF-I and/or IGF-II, and that these acted as autocrine, paracrine or intracrine agents to promote breast cancer growth (29-35). Despite the attractiveness of these hypotheses, such mechanisms have yet to be established conclusively. Data from the original laboratories and others (36-39), and from our group (10), have raised serious questions concerning participation of these growth factors in estrogen-dependent growth.

Our recent work supported by US Army grant DAMD17-94-J-4473 has now resolved this issue. We have discovered that two well known and thoroughly characterized plasma glycoproteins, SHBG and CBG, are negative regulators (*i.e.* inhibitors) of estrogen receptor positive (ER$^+$) and progesterone receptor positive (PR$^+$) breast cancer cell growth, respectively, and that autonomous (ER$^-$ and PR$^-$) breast cancer cells are not inhibited by these glycoproteins. The experimental results describing these studies are part of a series of nine manuscripts in preparation (see the listing below)

SHBG, which is a M_r 94,000 homodimer (40,41), has been identified as the major plasma carrier of sex hormones (42-45). CBG is a M_r 58,000 monomer which serves that function for progestins and corticosteroids (42). These proteins appeared to have no function beyond regulating the relative levels of "free/active" versus "bound/inactive" hormones in plasma (42,46). Evidence suggesting direct cellular functions became apparent only after the identification of SHBG receptors on sex hormone target tissues (47-57) and CBG receptors on human breast cancer and other target cells (42,58,59,61). Nevertheless, the function of these sites has remained unexplained. Studies have asked if these receptors might be docking sites for delivery of steroid hormones to the interior of cells. However, further work suggests this is unlikely (42,49). More positively, the receptors appear to possess a transmembrane signaling function which implies a regulatory role. When hormone free SHBG or CBG are bound to their receptors, the association of an appropriate steroid with the bound ligand induces a transient increase in cyclic AMP production (42,55,59,60).

Most of what is known about SHBG and CBG receptors has come from measuring the binding of ^{125}I-labeled SHBG or ^{125}I-CBG to whole cells, membrane fractions of cells or detergent solubilized membranes. The solubilized SHBG receptor from prostate cells showed an ^{125}I-SHBG affinity of K_a ~3 x 10^{10} M^{-1} (54,55). Studies with ^{125}I-CBG and human breast cancer MCF-7 cells gave K_a of ~ 1.4 x 10^9 M^{-1} (42,59). Studies have estimated the M_r of the SHBG receptor at ~ 170,000 (52,54). Another study showed that the amino acid residues 48→ 57 of SHBG contained a receptor recognition sequence (56). Beyond this information however, not even partial amino acid sequences are available for these receptors.

To place our study in perspective, it is vital to recall that the loss of negative regulation already has been implicated in a striking increase in susceptibility to breast cancer. For example, the autosomal dominant BRCA1 gene appears to code for a tumor suppressor protein (62-64). Mutations in BRCA1 confer a risk of 85% to breast and ovarian cancer. This autosomal dominant trait is carried by about 5% of the women in the United States. Also, the loss of the function (*i.e.* mutation) of the p53 tumor suppressor protein has important implications for germline and sporadic (*i.e.* non-familial) breast cancer susceptibility (65-68).

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C. Purpose of the Present Work

Shown below is our original Statement of Work. The purpose of the next year will be to continue with the primary goal of our original proposed research, namely, to structurally define the serum factor(s) which regulates steroid hormone dependent breast cancer cell growth. While these studies already have yielded remarkable new insights, they have also expanded Task 1 to include additional purification work and more cDNA cloning and DNA sequencing. Before our discoveries this past year, SHBG and CBG had never been implicated in breast cancer growth. Indeed, to make matters even more interesting, the form of SHBG we have isolated was completely unexpected. *These discoveries bear directly on our original theme of the negative regulation of breast cell growth.*

G. STATEMENT OF WORK

TITLE: ESTROGEN RESPONSIVE BREAST CANCER GROWTH REGULATION

TASK 1. Purification of Estrogen Mediating Serum Factor (EMSF): Months 12-30

    (a) Purification will be done by application of conventional, FPLC and HPLC methods using horse and, if indicated, human serum.
    (b) N^{α} and internal amino acid microsequencing will be done.
    (c) Computer registered sequences will be used to identify EMSF.
    (d) Rabbit polyclonal antisera will be raised against ovalbumin conjugates of EMSF peptides synthesized by solid phase methods.
    (e) Immunoadsorption will be used to confirm the relationship between amino acid sequence and activity.
    (f) The tissue of origin of EMSF will be sought in mouse organ extracts by Northern blotting for m-RNA.
    (g) The effects of estrogen status on EMSF concentration in mouse blood will be assessed by ELISA or RIA.
    (h) cDNA libraries will be screened for EMSF containing plasmids.
    (i) If the complete sequence of EMSF is not known, we will determine it by dsDNA dideoxy chain termination sequencing.

TASK 2. Characterization of the EMSF Receptor: Months 12-48

    (a) ^{125}I-EMSF will be made by the chloramine T reaction.
    (b) Specific ^{125}I-EMSF binding will be investigated with estrogen sensitive breast cancer cells. The experimental parameters will be time, concentration, temperature, and effects of estrogens in the culture medium. Scatchard analysis is expected to give K_D values and numbers of sites per cell.
    (c) Chemical cross-linking with ^{125}I-EMSF followed by SDS-PAGE and autoradiography will be used to identify the M_r of the specific ligand binding receptor.
    (d) Internalization will be studied to determine if EMSF undergoes receptor mediated endocytosis.
    (e) The same studies outlined in (a) will be done to determine if estrogen insensitive breast cancer cells lack EMSF specific binding.
    (f) MAbs will be raised which block ^{125}I-EMSF binding to receptors.
    (g) The Mabs will be used to screen cDNA libraries from estrogen sensitive cells to obtain plasmids for dideoxy dsDNA sequencing of the complete receptor.
    (h) Transfection with full length receptor cDNA will be evaluated to determine if estrogen insensitive cells can be reverted to negative control by EMSF.

TASK 3. Physiological Significance in vitro and in vivo: Months 30-48

    (a) The MAbs raised will be evaluated as growth agonists and antagonists in serum-free culture with estrogen sensitive breast cancer cells.
    (b) The effects of antagonistic MAbs will be investigated on estrogen sensitive and insensitive breast cancer cell tumor formation athymic nude mice.

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Human SHBG has been thoroughly characterized. It is purified from pregnant human female plasma or from female plasma by androgen (testosterone) affinity chromatography. This kind of SHBG is known in many species. We have tentatively designated it *Type I human SHBG* based on its amino acid sequence and its high affinity for sex steroid hormones. We have isolated a new form of SHBG from horse serum and rat serum. This new type does not share high sequence homology with the testosterone affinity purified SHBGs from human and rabbit. In fact, the homology is at best 30 to 40%. Also, horse and rat SHBG bind androgens and estrogens with a ten-fold lower affinity than Type I human SHBG. The marked contrasts in primary structure apparently cause differences in antigenicity and steroid hormone binding. These variant characteristics may explain why Type II SHBG has not been identified before.

Based on these new discoveries, the statement of work for the next year is described next:

CONTINUATION TASKS FOR THE UPCOMING NEXT YEAR (1995-1996):
(1) We will continue the identification of Type II SHBG by performing cDNA cloning and sequencing of the horse and rat proteins (an expanded form of original tasks 1h and 1i).
(2) We will use both biochemical isolation and molecular cloning methods to seek human Type II SHBG (part of original task 1a).
(3) We will develop immunological methods of measuring Type II SHBG in rat plasma and human serum samples (part of original tasks 1d and 1e).
(4) We will determine the endocrine physiology/hormone regulation/tissue of origin of rat serum SHBG (part of original tasks 1f and 1g).

D. Methods of Approach

(1) tasks 1(h) and 1(i) --- molecular cloning and cDNA sequencing of horse SHBG and rat SHBG: Our partial amino acid sequencing of horse SHBG shows it to be only 30 to 40% homologous to human or rabbit SHBG. Although this certainly is a relatively abundant and inexpensive source of SHBG (and CBG), to conduct effective receptor research it is very useful to know the complete amino acid sequence of the ligand. We will do this by cDNA cloning. We will use a horse liver λZAP library obtained from Texas A & M. If full length SHBG cDNA cannot be found in this library, fresh horse liver will be used to prepare poly (A^+) RNA for a new λZAP cDNA library by the methods described (69). These expression libraries will be screen for SHBG cDNA with rabbit antibody against human SHBG. It cross reacts with the horse protein although only at relatively low dilutions. The antiserum is available commercially. In addition, we

are developing a polymerase chain reaction (PCR) strategy for cloning horse SHBG. From the partial amino acid sequence obtained already, oligonucleotide probes were prepared to amplify specific intervening regions of the mRNA from horse liver poly (A$^+$) RNA. This amplified cDNA will be ^{32}P labeled and used in hybridization screening. This is a newer approach not considered in our original application. Also, rat SHBG in plasma has never been identified. We have now purified a significant supply. If partial amino acid sequencing confirms that rat SHBG is significantly different than SHBGs already reported (70,71), we will conduct the same cDNA cloning study of rat SHBG to determine its complete amino acid sequence. A rat liver library in λZAP is available commercially from Stratagene. Since our original application, we have a new fluorescence automated DNA sequencer which will be used in place of the previous manual methods. It determines ≥250 bases in a single analysis.

These studies are not necessary with horse CBG because our partial amino acid sequencing shows ≥ 90% homology to the human protein. Also rat CBG has been sequenced previously. The rat CBG isolated by us shows an identical partial sequence to that already published.

(2) work added to TASK 1 by our experimental results--- isolation and cDNA cloning of human Type II SHBG. *We emphasize again that the discovery of a Type II form of SHBG in other species has led to our search of human serum for a similar form. This is an unexpected addition to the project, but certainly one which is central of the original goals of the research program.*

We plan to purify human Type II SHBG from male plasma. Western analysis suggests that males may have relatively higher levels of Type II than females. Indeed, the level of Type II may be lowest in pregnant female serum, which is most often used to isolate SHBG. To purify human Type II SHBG, we will again use the cortisol affinity column method. However, before this is done, Type I SHBG (which has a higher affinity for androgens) will be removed by testosterone affinity chromatography. Additional purification will be achieved by hydrophobic interaction chromatography under various conditions which are expected to separate the Type I and Type II forms.

After completing the purification, we will conduct partial amino acid sequencing by our current methods. The sequences obtained will be used to prepare antibody and oligonucleotide probes for cDNA cloning from human liver libraries. The methods to be employed are described above with horse and rat Type II SHBGs.

(3) tasks 1(d) and 1(e) --- immunological reagents for specifically measuring human Type II SHBG in plasma samples and rat Type II SHBG in rat plasma: The methods for raising rabbit polyclonal antibodies against peptides corresponding to the amino acid sequences of rat SHBG have been described in detail in our original application and will be applied without modification.

For human Type II SHBG, we will use this same methodology with peptides which are clearly different in Types I and II SHBG. This is expected to yield antisera which specifically recognize one or the other of these two forms. If specific identification cannot be achieved by this method, we will use both Type I and Type II human SHBG to raise distinguishing mouse monoclonal antibodies by the methods described in our original application.

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        (4) tasks 1(f) and 1(g) --- endocrine physiology/hormone regulation/tissue of origin of rat serum SHBG: These studies described in our original application will be carried out with only one change. Instead of using mice as our experimental animal, we will use rats. All other methods and procedures will be used without modification. The change in animal model is being made because of our discovery of rat serum Type II SHBG. This change does not in any way alter our original goals.

III. BODY OF THE PROGRESS REPORT

A. Manuscripts in Preparation

The manuscripts listed next are in various stages of preparation and drafting:

(1) Moreno-Cuevas JE & Sirbasku DA. Steroid hormone-responsive growth of the MTW9/PL2 rat mammary tumor cells in culture medium supplemented with charcoal-dextran extracted serum. (To be submitted to *Molecular and Cellular Endocrinology*)

(2) Moreno-Cuevas, JE & Sirbasku DA. Estrogen-dependent growth of the MTW9/PL2 rat mammary tumor cells in culture. Evidence for a role of a calcium related inhibitory serum protein. (To be submitted to the *Journal of Steroid Hormone Biochemistry and Molecular Biology Molecular*).

(3) Tanji, M, Moreno-Cuevas, JE & Sirbasku DA. Purification and characterization of sex hormone-binding globulin (SHBG) and corticosteroid-binding globulin (CBG) from equine serum. (To be submitted to *Endocrinology*).

(4) Tanji, M, Moreno-Cuevas, JE & Sirbasku DA. Properties of sex hormone-binding globulin (SHBG) and corticosteroid-binding globulin (CBG) purified from adult rat serum by cortisol-agarose and phenyl-Sepharose chromatography. (To be submitted to *Endocrinology*).

(5) Moreno-Cuevas JE & Sirbasku DA. Identification of sex hormone-binding globulin (SHBG) receptors on MTW9/PL2 rat mammary tumor cells. (To be submitted to *Biochemistry and Biophysics Research Communications*).

(6) Moreno-Cuevas, JE, Tanji M & Sirbasku DA. Identification of sex hormone-binding globulin (SHBG) as the serum factor which regulates estrogen-responsive MTW9/PL2 rat mammary tumor cell growth in culture. (to be submitted to *Endocrinology*).

(7) Moreno-Cuevas JE & Sirbasku DA. Sex hormone-binding globulin (SHBG) regulation of estrogen-responsive MTW9/PL2 rat mammary tumor cell growth in serum-free hormonally defined medium. (To be submitted to the *Proceeding of the National Academy of Science, USA*).

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(8) Moreno-Cuevas JE & Sirbasku DA. Identification of corticosteroid-binding globulin (CBG) receptors on MTW9/PL2 rat mammary tumor cells. (To be submitted to *Molecular and Cellular Endocrinology*).

(9) Moreno-Cuevas JE & Sirbasku DA. Corticosteroid binding-globulin (CBG) regulation of progesterone stimulated MTW9/PL2 rat mammary tumor cell growth in serum-free hormonally defined medium. (To be submitted to *Endocrinology*).

B. Results Obtained During the First 12 Months

We have completed parts of tasks 1(a) through 1(c) and tasks 2(a) and 2(b). These initial studies are summarized next as abstracts of the results to be published in nine manuscripts.

(1) Manuscript 1: The MTW9/PL cell line was established in culture from the carcinogen-induced hormone-responsive MT-W9A rat mammary tumor and shown to form estrogen, androgen and progesterone responsive tumors in Wistar-Furth rats. The MTW9/PL line was used to derive the MTW9/PL2 cells which also exhibit estrogen-responsiveness *in vivo*. In this study, we describe serum supplemented cell culture conditions in which the MTW9/PL2 cell line demonstrates steroid hormone growth responsiveness. The serum used in the cell cultures was steroid hormone-depleted by charcoal-dextran treatment at $34°C$. When increasing concentrations of hormone-depleted serum were added to MTW9/PL2 cell cultures, growth was inhibited progressively. Between 30 and 50% (v/v) hormone-depleted serum suppressed growth completely. Addition of 1.0×10^{-13} M to 1.0×10^{8} M 17β-estradiol (E_2) reversed the serum dependent inhibition. Under optimal conditions, estrogen induced growth was 2^5 to 2^6 or 32 to 64-fold greater than in control cultures. Even when the cells had been inhibited for six days, E_2 addition restored growth. At 1.0×10^{-11} to 1.0×10^{-7} M, estrone, estriol and the synthetic estrogen diethylstilbestrol promoted optimal growth. Testosterone and dihydrotestosterone were nearly as effective but only at $\geq 10^{-7}$ M. Progesterone was partially effective at $\geq 1.0 \times 10^{-6}$ M. Cortisol was only marginally effective. MTW9/PL2 cells have high affinity receptors for estrogens and progesterone but not androgens. The serum from the adults of several species including rat and human demonstrated activity. Fetal bovine and fetal equine serum showed little activity. The studies described show that the same pattern of hormone-responsiveness seen with MTW9/PL cells in rats is now demonstrable in culture. Furthermore, the MTW9/PL2 cells in culture are the most steroid hormone growth responsive of any of the rat mammary tumor lines yet reported.

Because this cell line so very closely mimics those of human breast, it was used for all of our initial studies of the serum factor identity as SHBG and CBG. Key results were checked with the estrogen and progesterone responsive T47D human breast cancer cell line. This approach saved about six moths during the first year. The rat MTW9/PL2 bioassays require 7 days. The T47D assays required 14 days.

2. Manuscript 2: The purification of the serum factor mediating the steroid hormone effects on breast cancer cells initially was a very perplexing problem. The factor(s) was completely stable in whole serum for three weeks at 4°C, and yet was remarkably unstable during purification steps lasting only one week. Approximately three months of frustration were invested before we found that simple dialysis caused inactivation. Ultrafiltration proved that the factor was not lost in the dialysate, and therefore, must be a high molecular weight entity. Further exploration revealed that the "factor" was two activities. Their M_r were 200,000 and 55,000. Both were acid and urea labile, and lost when heated at 60°C. Chelex treatment caused inactivation, which suggested metal ion stabilization. A study of metal ions revealed that calcium at 0.1 to 50 mM stabilized the activity. Other metals were not effective. From these constellation of properties, we thought that the activity in serum might be SHBG. Western immunoblotting with anti-human SHBG confirmed that the activity and SHBG coincided in the same fractions from molecular sieve chromatography. However, there were two major dilemmas which contraindicated SHBG. First, we were using horse serum for these studies. Horse serum was not thought to have SHBG. Also, we were assaying with rat mammary cells. Adult rats were not thought to have plasma SHBG. Therefore, we concluded that the calcium stabilized factor(s) was SHBG related immunologically, but was not functionally SHBG.

3. Manuscript 3: Dr. Masao Tanji is an excellent protein chemist trained in this technology in the very prestigious Department of Biochemistry and Biophysics at the University of Tokyo. We are pleased he has come to Houston and joined our group. He analyzed our data and concluded that SHBG in horse serum might be present, but that it had different properties than SHBG from humans or rabbits. His analysis proved correct. In this manuscript in preparation, Dr. Tanji has purified horse serum SHBG and CBG by a simple two step method. This method yielded 5 to 8 mg of SHBG and 26 to 30 mg of CBG per two-liters of horse serum. The molecular size and subunit structure of the two horse proteins was examined by Superdex chromatography, ultracentrifugation and SDS-PAGE ± reducing agents. The results were consistent with SHBG and CBG. Both proteins were investigated for immunological cross reactivity with anti-human SHBG and anti-human CBG. They cross reacted weakly but specifically by Western analysis. They were examined for steroid hormone binding properties. Horse CBG bound cortisol and progesterone but not dihydrotestosterone or estradiol. Horse SHBG bound dihydrotestosterone and estradiol but not cortisol or progesterone. The partial amino acid sequencing of horse CBG confirmed a high degree of homology with human, rat and rabbit CBGs. The same analysis of horse SHBG showed only 30 to 40% amino acid homologies. We now plan to determine the complete amino acid sequence of horse SHBG. This is an exciting study because the differences and homologies between SHBG from different species can be used to understand such domains as the steroid hormone binding site and the domain(s) which participates in receptor binding. We also have studied the subunit structure of horse SHBG. This was done to gain an additional understanding of how SHBG might interact with cell surface receptors. SHBG has been described as a dimer of identical monomers. Nonetheless, the SDS-PAGE results with SHBG from horse serum showed subunits of M_r 60,000, 52,000 and 25-27,000. Our studies using chemical cross linking followed by ultracentrifugation and SDS-PAGE show that the molecule exists as three dimers representing the possible permutations of the two larger subunits. Furthermore, the 27,000 subunit is actually the two ends of a 52,000 subunit with a

proteolytic cleavage in the 164→ 188 disulfide loop. This cleavage divides the molecular approximately into halves. As yet, we do know the significance of the subunits nor do we know if the cleavage in this loop has any biological meaning. **However, these studies revealed a potentially major discovery with regard to a new therapy for breast cancer**. The monomers of SHBG are irreversible inhibitors of breast cancer cell growth. Future expansion of this observation is planned and will be the subject of a new NIH grant proposal.

4. Manuscript 4: The issue of the existence of SHBG in adult rat serum has been resolved by our laboratory group. Rats have SHBG, but it is not highly homologous to human or rabbit SHBG. We purchased one liter of a mixture of adult male and female rat serum and used it to conduct the simultaneous purification of CBG and SHBG by the method of Fernlund and Laurell. This yielded 3 mg of CBG and 6 mg of SHBG. The M_r of CBG was 58,000. Only one subunit was found. A partial amino acid sequence of rat CBG was obtained by us and compared to the published structure. There was an exact match in 50 of 50 residues. Next, we studied the rat SHBG. Rat serum SHBG cross reacts with rabbit anti-human SHBG by Western immunoblotting. SDS-PAGE, sedimentation and molecular sieve chromatography confirm a M_r 110,000 dimer. The data support the view that the monomers are very similar. Three subunits of M_r 58,000, 52,000 and 25,000 were identified by SDS-PAGE. Using a filter binding assay, we have found that rat serum SHBG binds dihyrdotestosterone and 17β-estradiol, but not cortisol or progesterone. This property defines this molecule as a sex hormone binding globulin. Because rat is well known to express a SHBG-like protein in testis, named rat androgen binding protein (ABP), we compared our SHBG from rat serum to the known amino acid sequence of ABP. By the sequences we have obtained, there is only 50% homology between rat serum SHBG and rat testis ABP. Furthermore, rat serum SHBG is blocked in the amino terminus whereas ABP is not. As stated above, molecular cloning will be used to obtain the complete amino acid sequence of rat serum SHBG.

5. Manuscript 5: We have identified SHBG receptors on MTW9/PL2 rat mammary tumor cells in culture. Radio iodinated SHBG was prepared by a modification of the chloramine T method used by us before. We have established the temperature , time and concentration dependence of ^{125}I-SHBG binding. For these studies specific binding of radio labeled SHBG was measured as total binding minus binding in cultures containing a 300-fold excess of unlabeled SHBG. Binding at 37°C reached a maximum at 2 hours. At 23°C, a maximum was reached at 3 hours. Incubations at 4°C showed a maximum at 6 to 10 hours. At the two higher temperatures, maximum binding was followed by a rapid decline suggesting receptor mediated endocytosis. This was blocked at 4°C. Scatchard analysis indicated a K_d of 1.0 nM for SHBG binding with ~ 30,00 sites per cell. All of the studies described were done with ^{125}I-SHBG from which steroid hormone had been removed by charcoal treatment at 34°C. When labeled SHBG was saturated with DHT or estradiol before initiating the binding studies at 4°C, all binding to receptors was blocked. When saturating concentrations of dihydrotestosterone or estradiol were added to the incubation conditions at 37°C, specific binding also was blocked. The data obtained with MTW9/PL2 cells confirm that only steroid hormone depleted SHBG binds to receptors. When sex steroid hormone is bound to free SHBG, the ligand no longer associates with receptor.

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6. Manuscript 6: In this study, we returned to the characterization of the serum factor(s) regulating estrogen responsive MTW9/PL2 cell growth in culture. As stated above, we knew that the serum factor was calcium related and that horse serum had SHBG. Now, the question became, "is the serum factor regulating estrogen responsiveness actually SHBG". For this study, we first validated a filter membrane method of measuring the SHBG content of serum using ^{3}H-DHT. This assay was based on a stoichiometry of one mole of ^{3}H-DHT bound per mole of SHBG dimer. Using this method, we estimated the SHBG concentration of horse serum was 4 to 5 mg/liter. This level compared very favorably to that in human female serum and was higher than in human male. Next, we conducted a series of studies in which we compared the effects of various agents and denaturing conditions on the serum content of SHBG to the effects on estrogen responsive MTW9/PL2 cell growth in culture. First, we measured the SHBG content of serum after dialysis against 0.05 M Tris-HCl, pH 7.4. After 72 hours, all SHBG was lost as measured by the steroid hormone binding assay. This same dialyzed serum also no longer supported estrogenic effects. Based on the fact that calcium and dihydrotestosterone (DHT) bind to SHBG to form a very stable complex, we investigated separately the effects of calcium, DHT and the combination of these on both SHBG stability and the estrogen related activity in serum. To summarize our results, any conditions in which SHBG was inactivated as determined by ^{3}H-DHT also showed an proportional loss of growth regulating activity. Finally, in this report, we demonstrated that SHBG addition to cultures supplemented with 2.5% horse serum leads to a progressive inhibition of growth which was complete by 2.5 µg/ml. Estradiol completely reversed this inhibition thereby restoring growth to maximum rates. The data in this report support the conclusion that SHBG is a major factor in serum which regulates estrogen responsive growth in culture. Nonetheless, the data did not excluded the possibility that something else in serum might be required for SHBG to act as an inhibitor.

7. Manuscript 7: In this report, we extended our study of the regulatory effects of SHBG to completely serum-free conditions. The purpose of this approach was to identify SHBG effects in the complete absence of other potential co-inhibitors in serum. In a previous study, we had reported the development of a serum-free hormonally defined medium for MTW9/PL2 cells. This medium contained F12-DME supplemented with 0.5 mg/ml bovine serum albumin, 10 µM ethanolamine, 10 ng/ml selenous acid, 50 µg/ml apotransferrin, 10 µg/ml diferric transferrin, 10 µg/ml insulin and 1.0 nM T_3. Growth in this medium was logarithmic for 10 days with a cell population doubling time of ~ 30 hours. The addition of 10 nM 17β-estradiol (E_2) increased the growth rate by 10 to 15%. When SHBG was added, 1.5 to 2.0 µg/ml completely inhibited growth. This response was reversed in part by 1.0×10^{-13} M E_2. Addition of 1.0×10^{-10} M E_2 promoted optimum growth equal to the estrogen response seen in serum containing cultures. A series of investigations were done to show that horse serum SHBG did not associate with E_2 in culture medium under concentrations reached 500 nM. The experiments presented indeed have confirmed that SHBG is a potent inhibitor of estrogen responsive mammary tumor cell growth and that estrogens reverse the inhibition.

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8. Manuscript 8: The presence of CBG receptors on MTW9/PL2 rat mammary tumor cells was established by the same methods outlined above for SHBG (see **Manuscript 5**). These studies confirmed approximate 100,000 receptor sites per cell.

9. Manuscript 9: In this report, we have carried out the study of the effects of CBG in serum-free hormonally defined culture. The medium used was the same as in **manuscript 7**. The results of this study were remarkable. Both estrogens and progestins reversed the inhibition caused by CBG. Cortisol had no effect. From the data obtained, we can conclude that both SHBG and CBG are inhibitors of mammary cell growth and that CBG inhibition is different in that progesterone reverses the effect. This will be the first ever report of progesterone regulated breast cancer cell growth in serum-free defined culture.

IV. CONCLUSIONS

	A. MAJOR CONCLUSIONS AND IMPLICATIONS
(1) SHBG is a physiologically important negative regulator of estrogen responsive breast cancer cell growth. This discovery is important because it is the first to identify a specific serum-borne entity which demonstrates such a function.	
(2) CBG is another major physiologically important negative regulator of progestin and estrogen responsive breast cancer cell growth. This plasma glycoprotein has not previously been recognized as having a function in breast cell growth regulation.	
(3) Our laboratory has identified a new area of breast cancer research. We have established that breast cell growth can be blocked by physiologically available serum-borne inhibitors and that steroid hormone responsive cell types remain quiescent until stimulated by estrogens or progestins.	
(4) Our data raise the possibility that humans may possess a form of SHBG which has not been recognized before. This SHBG (Type II) may be the form responsible for the inhibition of breast tissue growth under normal physiological conditions. We consider it possible that the lack of this form of SHBG in females may be a major predisposing factor to breast cancer.	

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B. FUTURE WORK AND CHANGES TO BETTER ADDRESS THE PROBLEM

There is one problem which is reaching major proportions. Our ability to produce purified SHBG and CBG has become the rate limiting factor in this research. Using the conventional chromatography equipment we have available in the laboratory, these proteins cannot be produced in amounts sufficient to meet our needs.

We have consulted with companies who provide modern automated chromatography systems. Pharmacia has a FPLC (Fast Performance Liquid Chromatography) system which will solve our problem. It is designed to run as an automated preparative system which will produce purified SHBG and CBG at ten day intervals. This system requires about one-quarter of the man-hours we now devote to this effort.

The FPLC will be custom designed for our work. It has computer controlled pumps and valves which move solutions from one column to the next without operator attendance. This permits preparations to proceed overnight and on weekends with only minimal supervision. At present, more than one-half of our laboratory man-hours are devoted to the preparation of these two proteins. This most certainly is not cost effective. In fact, there has been a noticeable slowing of our progress in the last two months because we must stop to make these preparations rather than conducting the next level of experiments.

Mr. Jack Kirten, a Pharmacia bioengineer, is now helping us design an automated FPLC system. His estimate of the costs will be $27,000 to $30,000. This amount was not planned into our original application because we had not yet identified the serum factors. Because of this, we could not anticipate the preparative problems we now encounter daily. We are in great need of assistance in this very critical area. As soon as we have a complete/detailed cost estimate from Pharmacia, we will petition the US Army Command for additional funds to purchase this very important equipment.

V. REFERENCES

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    pp 143-146 -- Formaldehyde gel for electrophoretic separation of RNA and Northern blot
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    pp 211-215 -- Plating and screening of λgt10 and λgt11 packaged inserts.
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